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14. ABSTRACT

Prostate cancer (PCa) is the most common male malignancy and the second leading cause of cancer mortality in US. To date, the most difficult task for PCa management is to distinguish between indolent and aggressive castration-resistance PCa (CRPC), which emerges when prostatic epithelial cells undergoing a phenotypic change of epithelial to mesenchymal transition (EMT). The acquisition of EMT in PCa cells increases their invasive potentials leading to metastases. In this study, we unveil the unique function of miRNA-363 (miR-363), often down regulated in high-grade PCa, as a potent anti-EMT microRNAs (miRNA). Noticeably, although miR-363 belongs to the miR-106a-363 cluster, the rest of miRNAs in this cluster closely resemble the oncogenic miR-17-92 cluster. Also, no miR-363 homolog is present in the miR-17-92 cluster. Our work provides a mechanistic insight of miR-363 regulation within the miR-106a-363 cluster by interferon-induced protein with tetratricopeptide repeats 5 (IFIT5) characterized as a viral RNA binding protein. IFIT5 appears to coordinate with XRN1and specifically mediate miR-363 turnover but not other miRNAs in this cluster. IFIT5 is highly elevated in high-grade PCa. Thus, this study delineates a novel machinery of the turnover of a tumor suppressive miRNA within a cluster containing mostly oncogenic miRNAs.

15. SUBJECT TERMS

Prostate cancer, microRNA turnover machinery, Interferon-induced protein with tetratricopeptide repeats 5 (IFIT5), epithelial-to-mesenchymal transition (EMT), microRNA-363, exoribonuclease XRN1.

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INTRODUCTION

MicroRNAs (miRNA) are a large family of short sequence single-stranded noncoding RNAs, which has been shown to regulate approximate 60% protein-coding genes via post-transcriptional suppression by facilitating mRNA degradation, or translational inhibition. Many miRNAs have been identified to be associated with different stages of tumor development [1,2]. Based on the seed sequence of 2-7 nucleotides, miRNAs are grouped into different families for predicting the potential target gene(s); the function of miRNAs could be divided into onco-miRNAs and tumor suppressor miRNAs. However, only handfuls of them have been validated experimentally. In this study, we clearly identify a unique miR-363 that is able to inhibit EMT in prostate cancer (PCa) by targeting Slug/SNAI2 mRNA. Clinically, a reduced miR-363 expression is correlated with PCa malignancy.

In general, similar to most protein-coding genes, miRNA genes can be regulated at transcriptional or post-transcriptional level [3-7]. Unlike most eukaryotic protein genes, several miRNAs such as miR-106a-363 and miR-17-92 are clustered together to generate a polycistronic primary transcript, which further complicates the regulatory scheme of miRNA biogenesis because each individual miRNA derived from one cluster may have different functional roles as well as expression levels in any given cell or tissue. For example, miR-363 belongs to the polycistronic miR-106a-363 cluster containing six miRNAs (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363), located on chromosome X. Unlike the other five miRNAs with similar seed sequences and similar functions as the oncogenic miR-17-92 cluster [8-10], miR-363 has been implicated to play a tumor suppressor role in nasal-type natural killer/T-cell lymphoma [11], hepatocellular carcinoma and colorectal cancer [12,13]. In this study, we unveil a new post-transcriptional regulatory mechanism specific to miR-363 turnover by a novel protein complex-Interferon-induced protein with tetratricopeptide repeats 5 (IFIT5), and our results further support the tumor suppressive role of miR-363 in PCa cells.

IFIT protein family was first identified as a viral RNA binding protein [14], as a part of antiviral defense mechanisms by intervening viral replication and/or disrupting viral RNA translation in host cells. Among IFIT orthologs, human IFIT1, IFIT2 and IFIT3 form a complex through the tetratricopeptide repeats (TPR) to degrade viral RNA [15]. On the contrary, IFIT5 acts solely as a monomer and binds directly to RNA molecules via its convoluted RNA-binding cleft. In a recent study, IFIT5 has been shown to directly bind to endogenous cellular RNA with a 5'-end phosphate cap, including transfer RNA (tRNA) [16,17], which partially shared a structural similarity with the precursor form of small RNAs such as small hairpin RNA (shRNA) and primary or precursor miRNAs. This is the first time in the literature to demonstrate that IFIT5 is able to specifically recognize a unique structure in the precursor miR-363 (pre-miR-363) and can facilitate the recruitment of XRN1 to degrade pre-miR-363. Also, the expression level of IFIT5 is inversely correlated with that of miR-363 in PCa specimens. In addition, a significant elevation of IFIT5 is detected in several PCa cells expressing EMT phenotypes associated with invasiveness. Thus, IFIT5-XRN1 complex mediates a specific degradation of tumor suppressor miRNA from its cluster, which could provide a new understanding of miRNA biogenesis for other tumor suppressor miRNAs.

KEYWORDS

Prostate cancer, Interferon-induced protein with tetratricopeptide repeats 5 (IFIT5), epithelial-to-mesenchymal transition (EMT), microRNA-363, microRNA turnover, , exoribonuclease XRN1.

OVERALL PROJECT SUMMARY

MicroRNA biogenesis becomes more complicated when individual miRNAs derived from the same polycistronic cluster being processed and expressed at different level. MicroRNA-363 is derived from a miR-106-363 cluster which is highly resembled to an oncogenic miR-17-92 cluster in their seed sequences. However, no ortholog of miR-363 was found in miR-17-92 cluster. Hence, we determined to unveil a unique biogenesis machinery of miR-363 specific from the rest of miRNAs in the miR-106a-363 cluster.

In this project, we identified the tumor suppressor role of miR-363 by intervene the EMT process in several cancer cell lines including PCa, hepatocellular carcinoma and renal cancer (Fig. 1). Most importantly, we elucidated a unique miRNA turnover machinery composed of IFIT5 and XRN1. We discovered that IFIT5 is capable of regulating the specific turnover of miR-363 from miR-106a-363 cluster (Fig. 2) via recognizing the unique 5'end structure of precursor miR-363 (Fig. 3). Moreover, XRN1, a 5' to 3' exoribonuclease, appears to be the key enzymatic component in IFIT5-mediated pre-miR-363 turnover machinery (Fig. 4), and that the C-terminal TPR domains of IFIT5 protein is required for recruiting XRN1 to perform its exoribonuclease activity on precursor miRNA degradation (Fig. 5). Clinically, IFIT5 mRNA level is elevated in higher grade PCa tumors, and a positive correlation was observed between IFIT5 and several mesenchymal markers such as Slug, Vimentin and ZEB1. This evidence indicates IFIT5 may possess oncogenic potential contributing to the metastasis in several cancer types including PCa (Fig. 6). Overall, this study provides not only a new knowledge of miRNA biogenesis but also a potential application of miR-363 and IFIT5 as a therapeutic agent in preventing cancer metastases.

CONCLUSION

We have demonstrated the first time that IFIT5 is able to specifically recognize a unique structure in the precursor miR-363 and recruit XRN1 to degrade miR363 (Fig. 7). We have also shown that the significant elevation of IFIT5 is detected in several PCa cells undergone EMT leading to highly metastatic potential and the expression level of IFIT5 is correlated with that of miR-363 in PCa specimens.

KEY RESEARCH ACCOMPLISHMENTS

- Characterization of differential expression of miR-363 from miR-106a-363 cluster regulated by miRNA turnover.

- Dissecting tumor suppressive function of miR-363 in preventing EMT by targeting Slug/SNAI2 in PCa cells.

- Identification of the role of DAB2IP in regulating IFIT5 and XRN1 expression.

- Unveiling new mechanism of action of IFIT5-XRN1 complex responsible for the specific miR-363 turnover from miR-106a-363 cluster.

- The negative clinical correlation of IFIT5 and miR-363 in PCa malignancies.

PUBLICATIONS, ABSTRACTS, AND PRESENTATION

1. Review article: Lo, U., Yang, D., Hsieh, J.T. (2013) The role of microRNAs in prostate cancer progression. Trans. Androl. Urol., 2: 228-241.
2. Peer-reviewed article: Lo, U., Pong, R.C., Yang, D., Gandee, L., Zhou, J.C., Tseng, S-F., Hsieh, J.T. (2015) IFIT5 complex, a unique microRNA turnover machinery, leading to epithelial-to-mesenchymal transition in prostate cancer. Cell Res. (submitted).
3. AACR podium presentation: U. Lo, R.C. Pong, D. Yang, J. Zhou, S.F. Tseng & J.T. Hsieh (2015) The specific regulation of miR-363 turnover from miR-106a-363 cluster by IFIT5 complex leading to epithelial-to-mesenchymal transition (EMT). Podium Presentation, *The 2015 annual meeting of American Association of Cancer Research (AACR),* Philadelphia, PA, USA.

4. Abstract: U. Lo, R.C. Pong, D. Yang, J. Zhou, S.F. Tseng & J.T. Hsieh (2015) The role of IFIT5 in miR-363 turnover. Poster Session, *The 2015 Keystone Symposium of MicroRNAs and Noncoding RNAs in Cancer,* Keystone, CO, USA.

REPORTABLE OUTCOMES
IFIT5 levels as a potential new prognostic marker for cancer metastasis.

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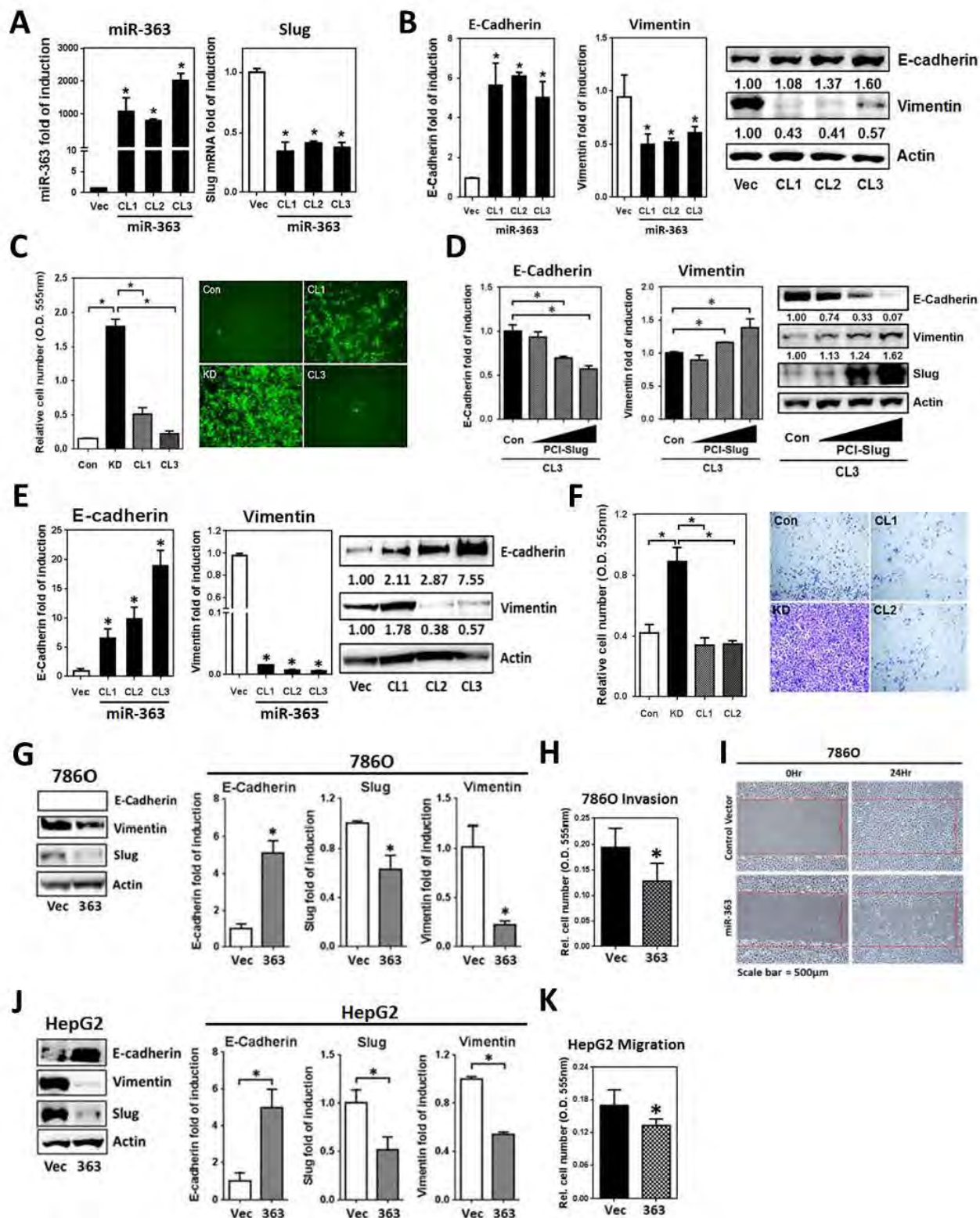**FIGURE 1. The effect of miR-363 on EMT in cancer cell lines.**

Figure 1. (A) Reduction of Slug/SNAI2 mRNA and protein levels in miR-363-expressing LAPC4-KD cells after normalizing with the control vector (Vec). (*p<0.05, CL: miR-363 expressing stable clone) **(B)** Expression levels of E-cadherin or Vimentin mRNA and protein in miR-363-expressing LAPC4-KD cells. **(C)** The effect of miR-363 on cell migration of GFP–expressing LAPC4-KD cells. GFP-positive cells were observed under microscope and migrated cells were stained with crystal violet and quantified at OD 555nm. Each bar represents mean ± SD of three replicated experiments. (* p<0.05). **(D)** The effect of Slug on the expression levels of Ecadherin and Vimentin mRNA and protein in miR-363 -expressing LAPC4-KD cells (CL3) after normalizing with the control vector (Con). (*P<0.05). **(E)** Expression levels of E-cadherin or Vimentin mRNA and protein in miR-363-expressing RWPE1-KD cells. **(F)** The effect of miR-363 on cell migration of RWPE1-KD cells. Migrated cells were stained with crystal violet and quantified at OD 555nm. Each bar represents mean ± SD of three replicated experiments. (* p<0.05). **(G)** Expression levels of E-cadherin or Vimentin protein and mRNA in miR-363-expressing 786O cells. **(H)** Transwell invasion of miR-363 expressing 786O cells. **(H)** Wound healing assay of miR-363 expressing 786O cells.**(J)** Expression levels of E-cadherin or Vimentin protein and mRNA in miR-363-expressing HepG2 cells. **(K)** Transwell migration of miR-363 expressing HepG2 cells.

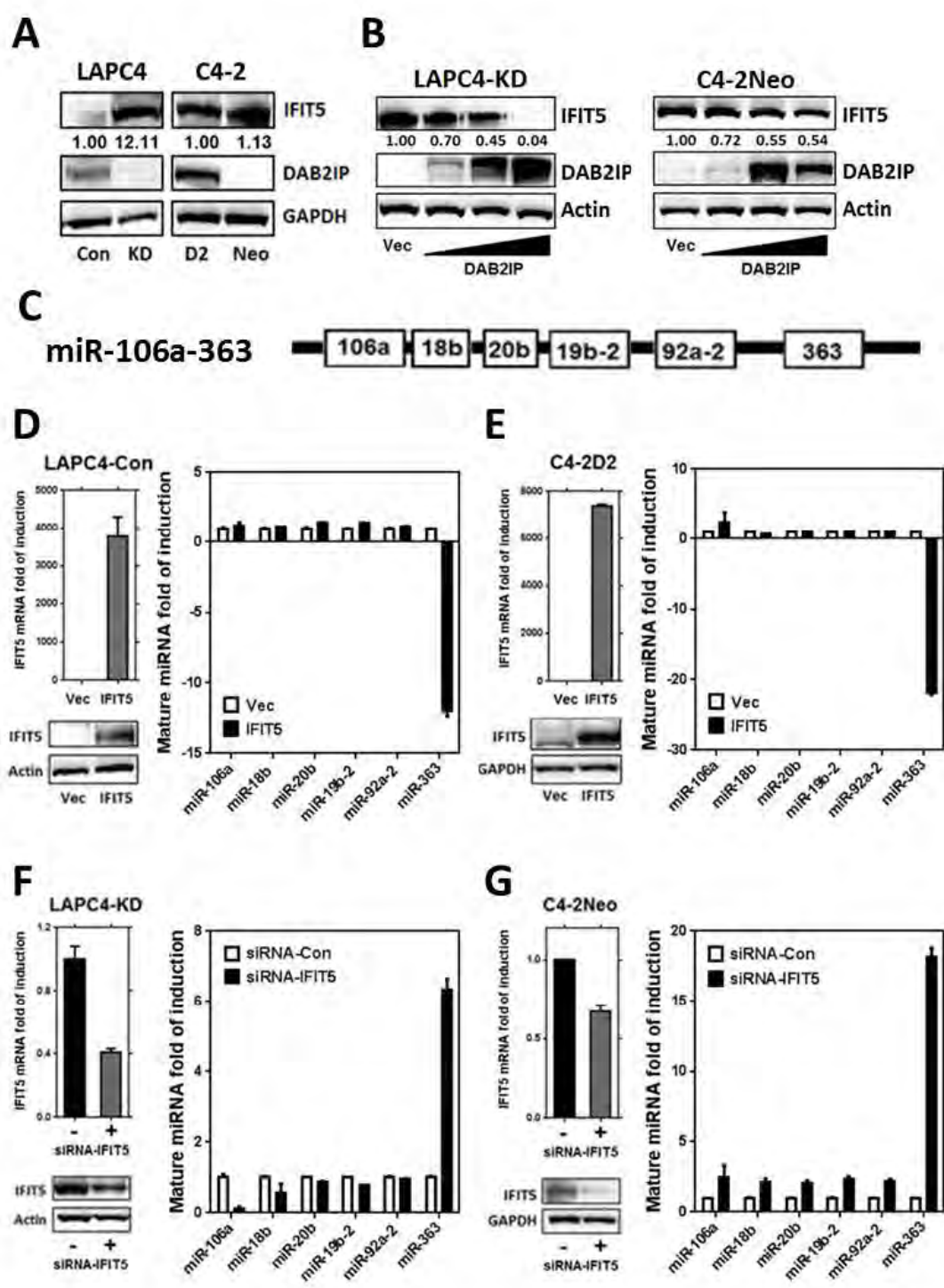**FIGURE 2. The impact of IFIT5 on miR-363 maturation from the miR-106a-363 cluster.**

Figure 2. (A) Expression level of IFIT5 in DAB2IP-positive and –negative lines (LAPC4, C4-2) **(B)** Ectopic expression of DAB2IP suppresses IFIT5 protein level in a dose-dependent manner in LAPC4-KD and C4-2Neo cells. **(C)** Schematic showing the position of each miRNAs in the miR-106a-363 cluster **(D-E)** Expression levels of mature miRNAs (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363) in IFIT5-expressing (IFIT5) LAPC4-Con and C4-2D2 cells after normalizing with the control vector (Vec). **(F-G)** Expression levels of mature miRNAs (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363) in IFIT5-KD (siRNA-IFIT5) LAPC4-KD and C4-2Neo cells compared to the control siRNA (siRNA-Con).

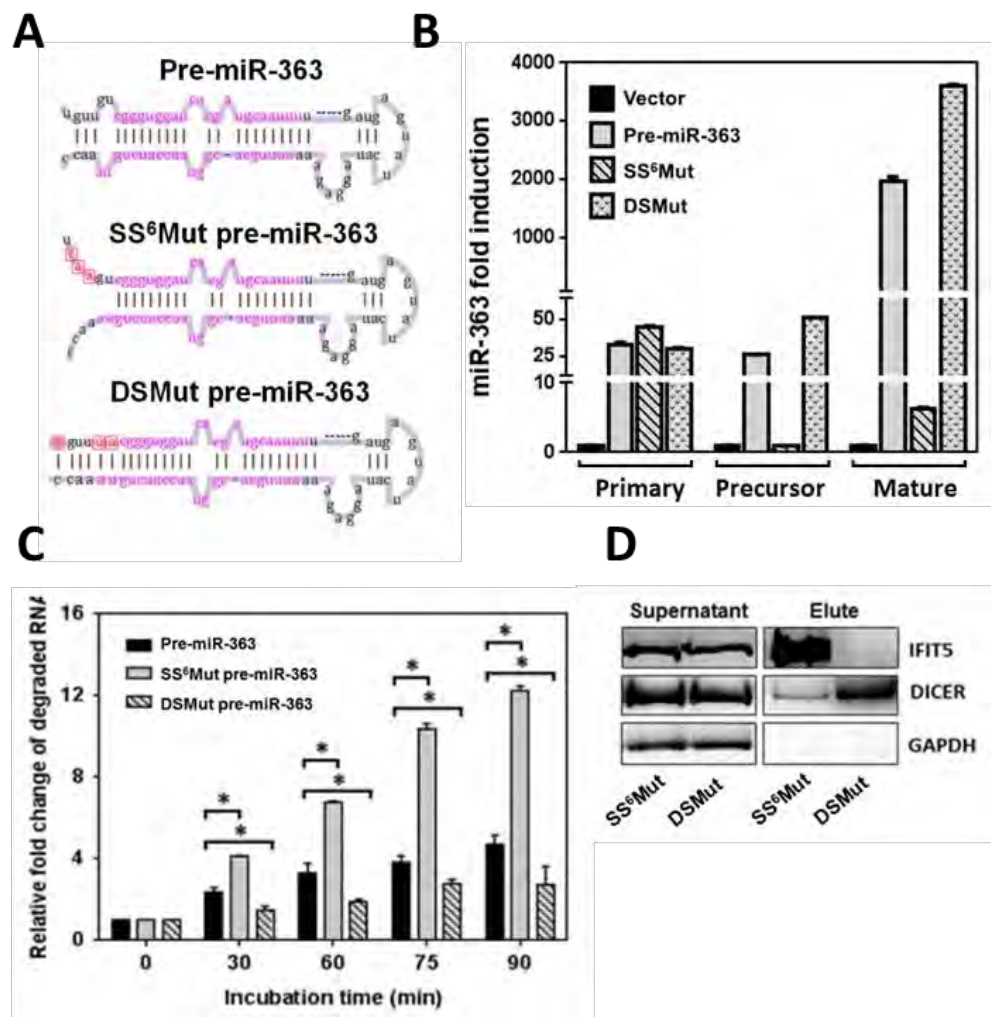**FIGURE 3. IFIT5-mediated precursor miR-363 degradation *in vitro*.**

Figure 3. **(A)** Upper panel: predicted structure and sequence of pre-miR-363. Middle panel: mutation of nucleotides (red box) for generating 5'-end single stranded pre-miR-363 (SS^{6}Mut pre-miR-363). Lower panel: mutation of nucleotides (red box) for generating blunt 5'-end double stranded pre-miR-363 (DSMut pre-miR-363). Both mature miR-363 and miR-363* sequence are shown in pink. **(B)** Expression levels of primary, precursor and mature miR-363 in LAPC4-KD cells transfected with Native, SS^{6}Mut or DSMut pre-miR-363 plasmids for 24 hrs after normalizing with the vector control. **(C)** Time-dependent change of degraded native, SS^{6}Mut and DSMut pre-miR-363 RNA fragments after incubation with immunoprecipitated IFIT5 protein at 37°C, each time point was normalized with 0 min. (*$p<0.05$). **(D)** Interaction between IFIT5 protein and SS^{6}Mut or DSMut pre-miR-363 RNA molecules using RNA pull down assay.

FIGURE 4. Interaction between XRN1 with IFIT5 leading to pre-miR-363 degradation *in vitro*.
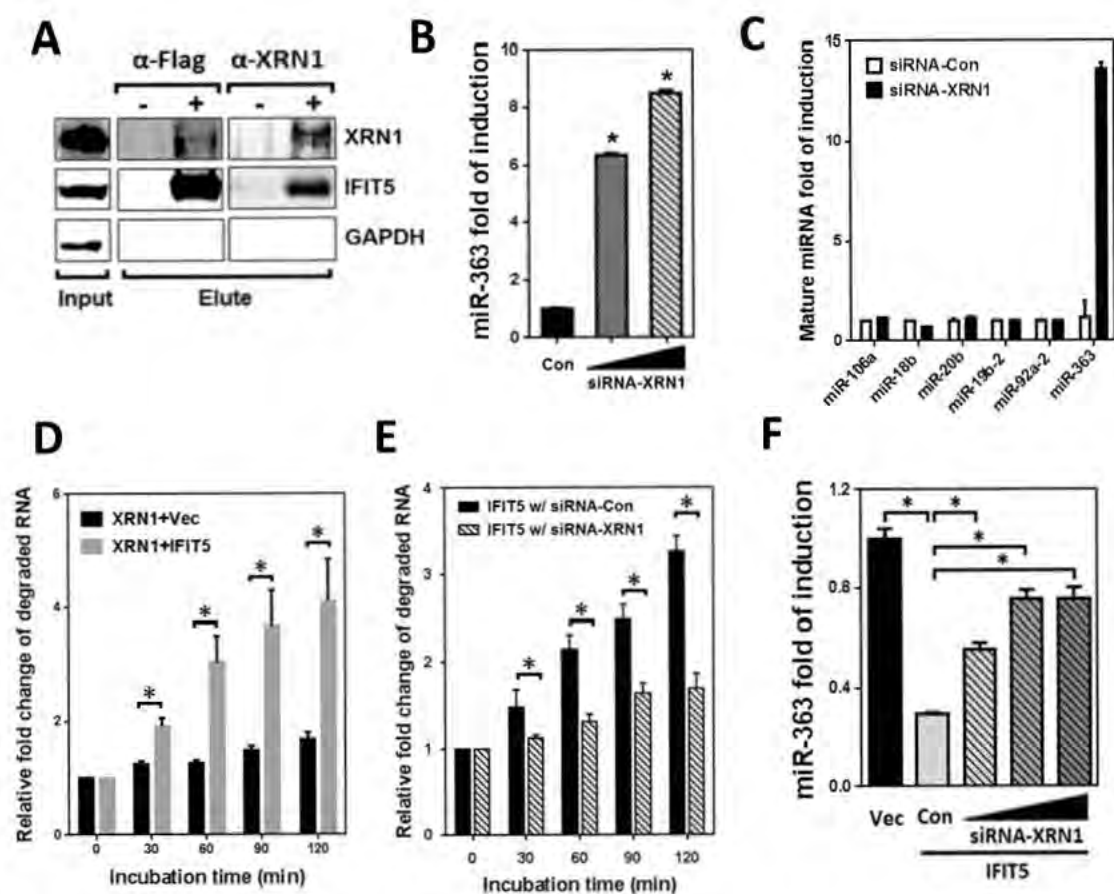
Figure 4. (A) Interaction between IFIT5 and XRN1 proteins using IP by Flag and XRN1 antibodies, respectively. **(B)** Induction of miR-363 expression in LAPC4-KD cells transfected with XRN1 siRNA and compared with the control siRNA (Con). **(C)** Expression levels of mature miRNAs (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363) in XRN1-KD (siRNA-XRN1) LAPC4-KD cells after normalizing with the control siRNA (siRNA-Con). **(D)** Time-dependent change of degraded SS^{6}Mut pre-miR-363 fragments after incubation with XRN1 alone (XRN1+Vec) or XRN1-IFIT5 complex (XRN1+IFIT5) at 37°C after normalizing with 0 min. (*p<0.05) **(E)** Time-dependent change of degraded SS^{6}Mut pre-miR-363 after incubation with the immunocomplex derived from cells transfected with IFIT5 and control siRNA (IFIT5 w/siRNA-Con) or XRN1 siRNA (IFIT5 w/siRNA-XRN1) at 37°C after normalizing with 0 min. (*p<0.05) **(H)** Dose-dependent recovery of mature miR-363 expression in IFIT5-expressing LAPC4-Con cells transfected with XRN1 siRNA after normalizing with the control vector (Vec) (Con: control siRNA, *p<0.05).

FIGURE 5. The C-terminal TPR 7-8 domain of IFIT5 required for the interaction with pre-miR-363 and XRN1.
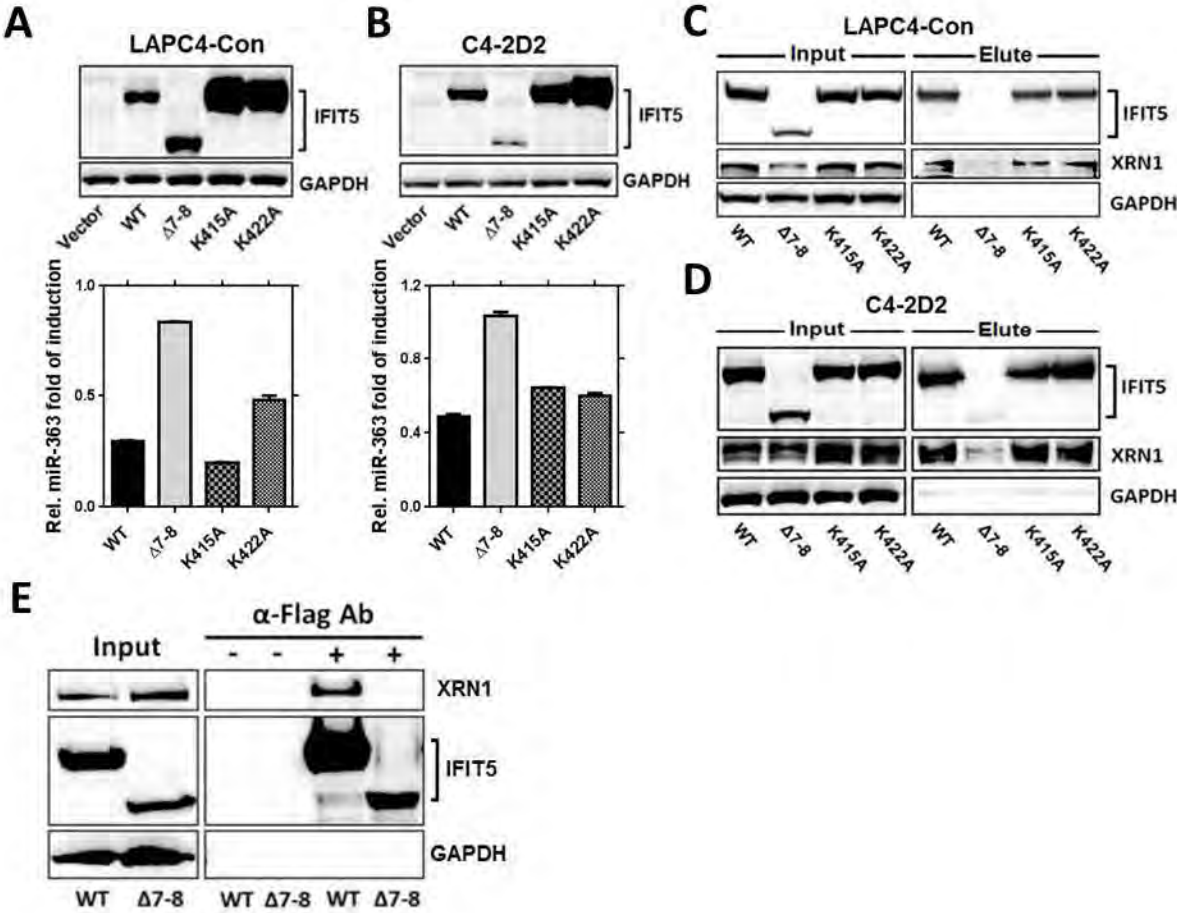
Figure 5. (A) Relative expression level of mature miR-363 in LAPC4-Con and C4-2D2 cells transfected with wild type (WT) and mutant (Δ7-8, K415A, K422A) IFIT5 after normalizing with the control vector. **(B)** Interaction between pre-miR-363 RNA molecule and wild type (WT) or mutant IFIT5 (Δ7-8, K415A, K422A) proteins derived from LAPC4-Con (Upper panel) or C4-2D2 (lower panel) cells using RNA pull down assay. **(C)** Interaction between XRN1 and wild type (WT) or Δ7-8 mutant IFIT5 derived from LAPC4-Con cells using IP. **(D)** The effect of SS[6]Mut or DSMut pre-miR-363 RNA molecule on the interaction between XRN1 and IFIT5 using IP with Flag antibodies.

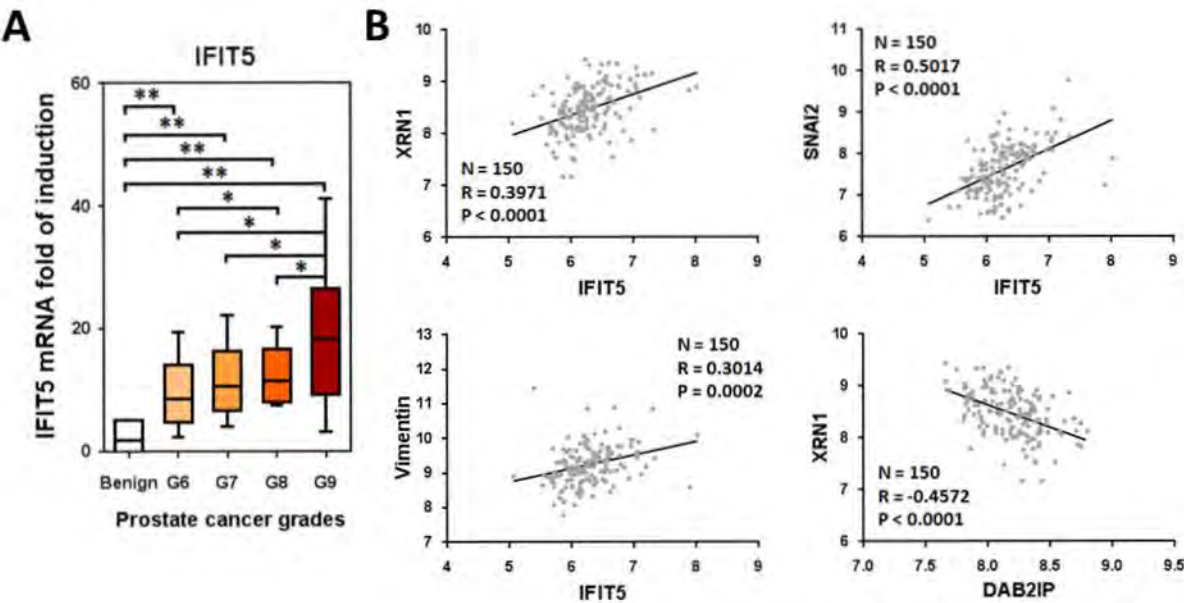**FIGURE 6. The clinical correlation between IFIT5 and EMT in PCa.**

Figure 6. (A) Relative induction of IFIT5 mRNA level in human PCa specimens including benign (N=10), G6 (N=9), G7(N=9), G8(N=6) and G9(N=7) (*p<0.05, **p<0.0001). **(B)** Clinical correlation between IFIT5 and XRN1, IFIT5 and SNAI2/Slug, IFIT5 and Vimentin, as well as DAB2IP and XRN1 mRNA level in PCa from TCGA PCa dataset.

FIGURE 7. Schematic representation of IFIT5-mediated pre-miR-363 turnover leading to EMT.